2006

Molecular methods for estimating soil microbial diversity in salt marshes

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MOLECULAR METHODS FOR ESTIMATING SOIL MICROBIAL DIVERSITY IN SALT MARSHES

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science-Biological Sciences,

Molecular Biology and Microbiology

by

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December 2006

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ABSTRACT

MOLECULAR METHODS FOR ESTIMATING SOIL MICROBIAL DIVERSITY IN SALT MARSHES

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This study represents the first attempt to estimate soil microbial diversity of salt marshes in the process of natural restoration. Our objective was to establish that soil bacterial diversity of salt marshes can be estimated using the PCR-RFLP method. The salt marshes in our study, Charleston Slough and Mountain View, are located in Mountain View, California. The bacterial 16S rRNA genes (1.5 kb) were PCR amplified from soil genomic DNA, and clone libraries were established for the respective marshes The 1.5 kb inserts were isolated from the recombinant clones and digested with *Msp*I and *Hae*III endonucleases. We were successful in obtaining 24 different RFLP patterns from a total of 45 clones obtained from soil samples of both the marshes. However, precise microbial diversity estimations require further optimizing the method and analyzing at least 200 clones from each of the marshes.

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisor, Dr. Sabine Rech, for her guidance, patience, and advice throughout this project. The time spent in her research lab has shaped me as a researcher and as an independent investigator. I would like to thank Joseph Dorado (lab member) for his evaluation of my presentations; and lastly, my lab mates for understanding my schedule.

I would like to thank my committee members: Dr. Steven White for constant patience in answering my questions and for many spontaneous lectures on the different aspects of the experiments; Dr. Ruthann Kibler for her interest, time, and patience in reading and providing valuable comments on earlier versions of this thesis.

I would also like to thank the entire SJSU Biology Department for their helpful discussions and technical assistance, especially Dr. John Boothby, Dr. Chris Brinegar, Dr. David Matthes, and Darcy Levee.

I also want to thank my mother Smt. Shanti Thanvi and my father Shri Fateh Krishna Thanvi for their constant support and encouragement during this journey. Most importantly I want to thank my husband Sanjay Vyas, for his patience, support, and for working with me throughout this document. I also want to acknowledge my son Keshav Vyas for adjusting without mommy every morning during my research work.

Finally, I want to thank Christina Peterson for her guidance on style and reference formatting.

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INTRODUCTION

1. Salt Marshes

Salt marshes are wetlands formed in the inter-tidal zones, where land meets the sea. They are exposed to tides periodically and are inhabited by grasses, herbs, and shrubs (Wenner 2006). Salt marshes are formed over hundreds of years by incoming tides. These tides bring in fine sediments and deposit them on the low-lying land. Slowly, salt marsh plants (e.g. *Spartina alterniflora*) begin growing on the sediment layers. During spring and summer the plants flourish, but during the fall season these plants start to die and eventually decay. The decayed plant material supports the growth of diatoms, bacteria, protists, and other organisms. These microorganisms are a source of food for many fishes, crabs, worms, and other animals. The feces of these animals serve as manure for the salt marsh plants of the next season (Estuary salt marshes 2006; Oceanside Meadows Institute 2002).

Marshes are mainly divided into two zones based on the amount of tidal water they receive. Marshes near the coast exposed to tides twice a day are called low marshes or inter-tidal marshes. Marshes away from the coast exposed to tides irregularly are called high marshes or upper marshes. High marshes remain without flooding for at least ten days (Mitsch and Gosselink 2000).

The salinity of water is the relative concentration of salt (predominantly sodium chloride) in a given water sample; nearly all water contains salts. Salinity of water is measured in parts per thousand (ppt), and the salinity of sea water is 36 ppt; i.e., 36

pounds of chloride per thousand pounds of water (Gore 2000). The salinity of salt marsh soil water can be as low as fresh water (2 ppt) or as high as sea water (36 ppt) (Pace 1999). Salinity depends on the frequency of tides, amount of rainfall, tidal creeks and drainage slopes, and type of soil. Lower marshes have constant salinity, equal to sea water because they are frequently flooded. Higher marshes, however, have fluctuating salinity because they are exposed to the atmosphere for longer periods of time and are irregularly flooded (Mitsch and Gosselink 2000).

The amount of rainfall also affects the salinity of marsh soil water. More rainfall leads to lower salinity, while drought leads to water evaporation, thereby increasing the salinity. Steep slopes drain away saline water thus reducing the salinity. Soils like clay and silts can hold more water, and therefore have higher salinity than sandy soil salt marshes (Mitsch and Gosselink 2000).

Vegetation in marshes is affected by salinity, amount of water accumulated – due to flooding – and the frequency and magnitude of flooding. Due to high salinity and anaerobic waterlogged soil the animal and plant population in salt marshes endure environmental stress (Wenner 2006).

Salt marshes are inhibited by halophytes, plants adapted to grow in a saline environment. There are four commonly found halophytic plant species in the salt marshes. Lower marshes are inhibited by tall (9 ft) halophytic grasses; *Spartina alterniflora,* commonly known as smooth cordgrass that can endure regular flooding and high salinity (Wenner 2006).

Higher marshes are less frequently exposed to tides and are colonized by shorter (3 ft), smooth halophytic grass; *Spartina patens,* known as marsh hay (Wenner 2006). This plant dominates in drier and saltier zones of the seaward side high marsh. *Juncus gerardi,* known as black needle rush, has a stiff, hollow stem and bears small flowers. These plants have very low salt tolerance and are found on the landward side of the higher marshes (Grasso 1999). *Distichlis spicata,* commonly known as salt grass, grows in hypersaline patches created in the marshes by some flotsam that destroy vegetation and where direct sunlight causes a lot of evaporation (Grasso 1999).

One other important halophyte found in salt marshes is *Phragmites australis.* It is a tall (up to 18 ft) invasive reed, commonly called phragmites reed. It grows in fresh and brackish water, but can also survive in acidic and alkaline environments (Maryland Sea Grant 2006). *Phragmites. australis* has a fast growth rate and propagates by seed dispersion, rhizomes, and stolon fragments. Due to their rapid growth, phragmites reeds have a monospecific stand (single species) in disturbed marshes. Disturbed marshes are those whose vegetation, water drainage, and soil nutrients are altered by natural intrusions, such as storms, lightning strike fires; or by human intrusions, such as logging, mining, waste disposal, intentional flooding, and dredge spoils disposal.

Marshes are present along the entire east coast of the United States, interrupted only by beaches along the Gulf of Mexico. On the west coast, however, they are found scattered from Washington to San Diego. Salt marshes located on eastern and southern coastlines of North America are dominated by cord grass. Higher marshes are dominated by *Spartina. patens*; whereas *Spartina. alterniflora* are dominant along channels and

ditches within the higher marsh, where regular flooding occurs; and *Phragmites. australis* flourishes along large areas of eastern coast line of United States, where the water is fresh to brackish (Mitsch and Gosselink 2000).

The Gulf Coast contains about 60% of the coastal marsh land of the entire United States (Fleury 2000). Along the Gulf Coast, where the water is warmer, a single species of black needle rush, *Juncus roemerianus*, dominates. However, *Spartina. alterniflora* forms the outer edge of the salt marsh. Around Hudson Bay, Alaska, Iceland, the northern British Isles, and Scandinavia, species of alkali grass, *Puccinell,* dominate the coastal salt marsh (Snyder 2001).

Southern California is a warm temperate region, and therefore high evaporation has a heavy influence on salinity and soil water quality. Here, grasses are much less abundant and are replaced by dicotyledonous halophytes. Widespread species with fleshy leaves or stems include the *Salicornia* species, pickleweed, *Suaeda californica*, saltwort, *Batis maritime,* and *Jaumea carnosa* (Snyder 2001).

Plants which carry out photosynthesis (synthesis of glucose and other food material) starting with 4-carbon molecules are called C4 plants. C4 species of high light environments can yield much higher photosynthetic rates at higher temperatures and lower water loss than their related C3 species, which are more typical of temperate and colder latitudes. In Southern California salt marshes, commonly found C4 species include *Salicornia, Suaeda, Atriplex, Distichlis,* and *Monanthochloe* (Snyder 2001).

Salt marshes also provide habitats for many animals, who cannot survive in other environments. Invertebrates such as fiddler crabs, marsh snails, worms, fishes, and

shrimps are present in marshes. Crabs, white shrimps, and spot-tail bass live in salt marshes where stems, leaves, and roots provide food and shelter from predators (Wenner 2006).

A large diversity of insects also feed on the salt marsh. They feed on living plants, detritus, and fluids secreted by living plants and play an important role in the food cycle. For example, many fishes and birds rely on insects as their source of food. Additionally, birds feed, perch, and reproduce in the salt marshes. Species like the red-winged black bird feed on insects and seeds; and egrets feed on fishes, crabs and shrimps. Clapper rails are among the bird species commonly seen foraging the salt marshes (Wenner 2006).

Despite stressful conditions, salt marshes are very productive ecosystems. One acre of salt marsh produces three times more biomass every year than one acre of cornfield. Salt marshes convert 6% of received sunlight into energy, due to the presence of a large number of phytoplankton and other producers that convert sunlight into chemical energy (Fleury 2000).

Gross Primary Production (GPP) is the total amount of carbon dioxide that is fixed by plants in photosynthesis. Some of this accumulated energy, however, is utilized by the primary producers themselves for respiration. Net Primary Productivity (NPP) is the net amount of primary production after the costs of plant respiration, which is measured in kcal or $g/m^2 \cdot y$ (Townsend and others 2003). The southern east coast marshes have a higher NPP than the northern marshes, because they are exposed to

sunlight for longer periods of time, and therefore have a longer growing season. The Gulf Coast has the highest NPP of 2000 $g/m^2 \cdot y$, because it has a year-long growing season (Barbour and Christensen 2004).

The ocean tides also help in the productivity of the salt marshes by bringing large amount of water, minerals, and nutrients, thereby flushing out waste matter that might hinder plant growth. Lower marshes have higher NPP, compared to higher marshes, because of more frequent tide inundation (Fleury 2000).

Salt marshes have additional beneficial qualities, like protecting the coast from erosion and removing debris. Roots of salt marsh plants hold the soil in place, reducing the wave's energy and thereby help prevent coastal erosion (Mitsch and Gosselink 2000; Yu and Mohan 2001). Organisms inhabiting the salt marshes decompose substances like industrial wastes, acids, and papers. The decomposed matter is then available as nutrients for other plants and animals (Mitsch and Gosselink 2000).

2. Salt Marsh Restoration

Salt marsh restoration means re-creating the original environment that harbors animals and plants, which cannot live in any other environment. Restoration efforts can be applied to the amount of water present in the marsh, salinity of the marsh, types of plants in the marsh, or a particular species in the marsh (Zedler 2001). Variation in water levels can be achieved by filling ditches and removing dikes, which exposes marshes to tidal action. Restoration of the plant vegetation is accomplished mainly by planting spartinas and removing invasive species (Zedler 2001).

A through understanding of the ecosystem process is very important for recreating the natural environment. A successful restoration is based on good scientific knowledge, clearly defined goals, systematic monitoring approach, an appropriate reference site, and long term management. The purpose of restoration has to be clearly and unambiguously defined before starting any restoration project. For example, restoration to specific salinity, turbidity, and dissolved oxygen standards of water, or restoration of a habitat for a particular species or group of species.

It is important to document plant, animal, and environmental conditions over the period of time to know the extent of success and problems in restoration. A reference site can serve as a model for the restoration site. Long-term management is required to ensure that the projects which are successful initially do not fail later (Zedler 2001). Additionally, before starting restoration projects, the following considerations should be evaluated. A considerable amount of money is required to fully restore the site. Many restoration sites are privately owned, so the owner's cooperation is needed. The restoration should be contained in the area of the marsh and should have minimal adverse effects on neighboring properties. Examples of adverse effects of restoring water include the harboring of insects, or localized flood risk increases if unintentional levee breaches occur (Zedler 2001).

3. Study Sites

Many California wetlands have disappeared due to diking, draining, and filling. Only 10% of the wetlands that existed in the 19th century still remain (California Coastal

Commission 1987). The damaged wetlands of California include the salt marshes in this study. Mountain View and Charleston Slough salt marshes are located in the Shoreline area of Mountain View, CA. Shoreline is a 700-acre wildlife preservation and recreation area owned by the city of Mountain View. It contains salt ponds, parks, trails, and salt marshes (Nisbet and others 2002). Figure 1 is a map showing the location of these two marshes in the Shoreline area.

3.1. Charleston Slough Salt Marsh

Charleston Slough salt marsh is located on the western side of the Shoreline area (Nisbet and others 2002). Before 1929, this marsh opened to the San Francisco Bay. In that year, however, a salt production company divided the Charleston Slough into an outer and an inner section by constructing a levee. The salt company also constructed two salt ponds, A1 and A2 (Figure 1). In 1980, the inner section of Charleston Slough salt marsh was acquired by the city of Mountain View. Since then, attempts have been made to restore this 30-acre marsh. The early restoration efforts were unsuccessful because of the poor water quality of the inner marsh. The salinity of the water fluctuated due to the sediment deposition in artificially constructed water channels; and algal blooms during summer produced an increased amount of dissolved oxygen, which was not conducive for salt marsh plants to flourish (Nisbet and others 2002).

The latest restoration effort of the Charleston Slough was initiated in 1997. The goals are to improve water quality, monitor water levels, sediment deposition, and grow pickleweed and cord grass for at least ten years. The city has built six culverts with tidal gates to control the incoming water. Current reports (Nisbet and others 2002) indicate

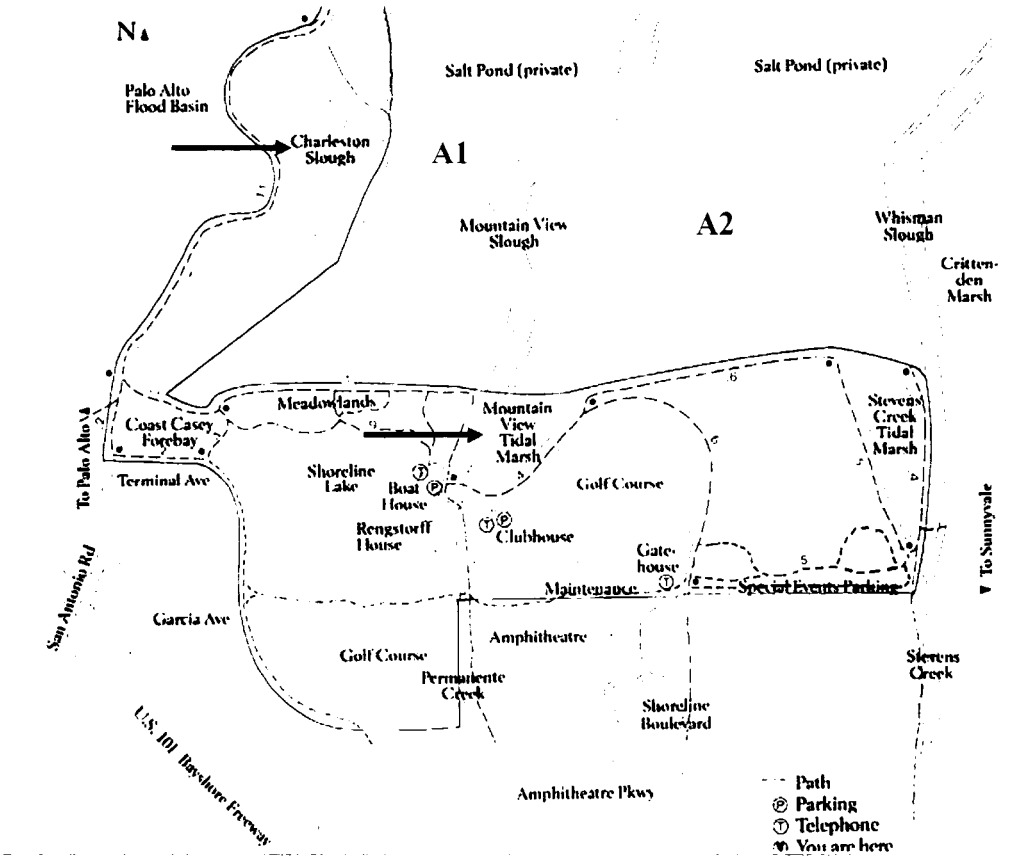

Figure 1. Map showing location of Mountain View and Charleston Slough salt marshes in the Shoreline area of Mountain View, California (The City of Mountain 2006).

that the Charleston Slough restoration project has gained success in improving water quality (less algal bloom, lesser salinity fluctuations, and low dissolved oxygen) (Nisbet and others 2002), the raised levees are still being maintained, and pickleweed is flourishing near the shoreline during the month of December. However, mudflats are still seen in more than 50% of the area (Nisbet and others 2002).

Charleston Slough marsh currently supports many bird species. Snowy plovers, black-necked stilts, and black rails nest on the levees, islands, and creek beds, especially during low tide, and ducks forage during high tides. Endangered species like the salt

marsh harvest mouse reside in pickle weed, and jackrabbits are also found in these marshes (Nisbet and others 2002).

3.2. Mountain View Salt Marsh

Mountain View salt marsh is located south of the A1 and A2 salt ponds (Figure 1) and encompasses approximately 12 acres. It was restored as a part of a mitigation project in early 1970s by the U.S. Army Corps of Engineers and completed in 1990 (Bay Area Wetland 2002). A survey (Shoreline, Mountain View 1995) shows that the restoration efforts were maintained, and no algal growth was seen on the surface of the water or on the mud during any season. Pickle weed grass flourishes all year long, and mudflats are limited.

A bird survey (Baker 2000) shows meadowlarks, red-winged black birds, sparrow, and egrets feed during low tide in winters. During the fall, hawks (northern harrier, turkey vultures), herons (snowy egrets), and other birds (logger head, shrike, ring necked pheasant, whistle crowned sparrow) are seen feeding, flying, perching, or nesting. From October through February many birds migrating from Canada to Mexico make their stop at the Shoreline marshes.

4. Microbial Diversity

Microbial diversity can be expressed by monitoring species richness and species evenness. Species richness is defined as the number of different types of species present

in a community, and evenness is defined as the number of organisms of each species present in a community (Kassen and Rainy 2004; Vigdis and Ovreas 2002).

Microbial diversity can be metabolic, morphological, or genetic (Ogunseitan 2005; Campbell, Reece, and Mitchell 1999). Metabolic microbial diversity is defined by differences in energy production, metabolism and biosynthesis of metabolites, which in turn can be used to classify microorganisms as autotrophs, heterotrophs, phototrophs, and chemoorganotrophs. Autotrophs produce organic molecules using carbon from carbon dioxide, and heterotrophs use other organic compounds as their carbon source for making organic molecules. Phototrophs use light as their energy source, and chemotrophs use chemicals as their energy source. Chemoorganotrophs use organic compounds as their energy source, and chemolithotrophs use inorganic compounds as their energy source (Campbell, Reece, and Mitchell 1999).

Morphological microbial diversity is defined by differences in the shapes of microorganisms. The common shapes of prokaryotes are spherical (cocci), rod (bacillus), or helical (spirilla). Microbial genetic diversity, in contrast, focuses on the comparison of conserved gene sequences. The most commonly used sequences are 16S rRNA gene sequences, which are often employed to construct phylogenetic trees (Daniel 1998). The rRNA genes, 5S, 16S, and 23S, carry conserved as well as unique sequences. The unique sequences can be species specific, group specific, and domain specific. Therefore, they are used to deduce phylogenetic relationship among microorganisms (Pace 1997, 1999). 16S rRNA gene sequences are more commonly used as target sequences because of their

larger size and sequence complexity than the other rRNA gene sequences (Pernthaler 2001).

Environmental conditions such as temperature, pH, available nutrients, and habitat complexity affect microbial diversity of a particular region (Holben 2003). A recent study by Yu and Mohan (2001) of microbial diversity of lagoons, for example, suggested low microbial diversity will exist under conditions of high temperatures and high concentrations of toxic organic compounds.

Traditional methods used to study microbial community involve isolating bacteria from the environmental samples and culturing them in the laboratory. A number of studies, however, suggest that 99% of microorganisms found in the soil cannot be cultured in the laboratory (Borneman and others 1999; Giller and others 1999). Traditional methods therefore cannot accurately estimate microbial diversity of a community (Rondon and others 2000). To solve this problem, culture independent techniques (including variety of molecular methods, fatty acid analysis etc.) have been developed to study microbial diversity (Dahllof 2002).

Developments in molecular phylogenetics have transformed microbial diversity studies in the last four decades. Researchers are now able to gather immense amounts of evolutionary information and map evolutionary diversification (Harrison 1968; Norman 1997). There are three approaches to study microbial diversity using molecular methods: (i) direct analysis using *in situ* methods; (ii) nucleic acid extraction to perform microarrays; and (iii) gene amplification by polymerase chain reaction (PCR) to perform pattern analysis, cloning, and sequencing, probe hybridization, or microarrays (Kassen

and Rainey 2004). The following briefly describes each of the major methods to estimate microbial diversity.

4.1. In Situ Methods

4.1.1. FISH (Fluorescent In Situ Hybridization)

FISH is a technique that involves the use of radioactively or fluorescently-labeled, short oligonucleotide probes, which are group-specific or species-specific. These oligonucleotide probes are designed using sequence information available from the databases and are hybridized to the desired target sequences (i.e. sequences encoding 16S rRNA). The signals are detected using either a fluorescent microscope (Pernthaler and others 2001; Rudolf, Bernhard, and Behrens 2001) or a flow cytometer (Fuchs and others 2000). Increased numbers of ribosomes in the bacterial cell lead to an increase in the fluorescent signal, which facilitates the signal detection (Pernthaler and others 2001).

The advantage of using this method is that direct analysis can be done without extraction of nucleic acid, which eliminates biases of nucleic acid extraction and PCR. This method, however, requires the presence of a high copy number of target sequences within an individual cell. Low fluorescent intensity is not detected by flow cytometery nor fluorescent microscopy, thus potentially giving false negative data (Dahllof 2002).

The number of target sequences present in the cell depends on its growth rate. More target sequences are present when the growth rate is high. Sometimes the target site may not be accessible to probes for FISH, due to the quaternary structure of rRNA or protection of rRNA segment by ribosomal proteins (Pernthaler and others 2001).

Moreover, FISH is used to detect the presence or absence of a particular group or species, because there are practical limits to how many probes and/or primer sets can be used to study the entire microflora of an environmental sample whose 16S rRNA sequences are not known (Kassen and Rainey 2004). Full 16S rRNA sequences are required for the probe to hybridize specifically. Partial sequence or no sequence information will reduce the potential target sites, and the specificity of the probes that target unsequenced regions of the 16S rRNAs will be questionable (Pernthaler and others 2001).

4.1.2. In Situ Polymerase Chain Reaction

This method was developed while studying HIV-1 infected cells passed from mother to fetus. The *in situ* PCR target can be any individual gene, mRNA, or rRNA. This means that genetic information, gene expression, and taxonomic information can be exploited. Prior to *in situ* PCR, cells or tissue are fixed and permeabilized to preserve morphology and allow PCR reagents access into the cells. Subsequently, *in situ* amplification is performed either in micro-eppendorf tubes using intact cells suspended in PCR reaction mixture or on glass slides, where tissue sections are placed with the PCR reaction mixture. In the former approach, a conventional thermal cycler is used for the reactions. After the PCR reaction is complete, the cells are cytocentrifuged onto a glass slide. Cytocentrifuging uses low-speed centrifugal force to separate and deposit a monolayer of cells on glass slides, while maintaining cellular integrity (Mengel 1985). The *in situ* PCR on glass slides is performed by overlaying the tissue section by standard PCR mixture (buffer, primers, polymerase, and nucleotides), covering the section with a

coverslip and sealing it with mineral oil. The slides are then placed on top of the heating block of a conventional or specially-designed thermal cycler. For signal detection, labeled nucleotides are used for both approaches during the PCR reaction (Hodson and others 1995). Hodson and others (1995) were the first to apply this technique to prokaryotic cells.

This technique can detect low copy genes and even single copy genes inside a cell, thus overcoming the limitation of the FISH technique. This technique, however, has only been used to detect the presence of known genes, mRNA, or rRNA sequences in environmental samples (Kassen and Rainey 2004). As an example, Hudson and others (1995) analyzed the presences of *amoA* gene in a microbial community of biofilms. The microbial cells were fixed, permeabilized, and then subjected to PCR with a digoxigenin labeled primer. The amplified fragments were detected by alkaline phosphatase-labeled antidigoxigenin antibody.

4.2. Whole DNA extraction

4.2.1. Microarray

Microarray, also called DNA chip technology, has recently been used in genotyping studies (Call, Brockman, and Chandler 2001). Whole cell DNA or RNA is extracted, followed by labeling of the extracted nucleic acids. Alternatively, PCR amplification of 16S rRNA sequences is performed, followed by labeling of PCR products. Oligonucleotide (a short, single chain of nucleotides, usually consisting of 20 nucleotides) arrays are synthesized on a solid support. Labeled nucleic acid or PCR

products are hybridized to the microarray, unbound nucleic acids are washed away; and hybridization analysis is performed (Call, Brockman, and Chandler 2001; Kassen and Rainey 2004). To map microbial diversity of a community using a microarray, the total RNA of the microorganisms of interest is extracted and labeled. The labeled RNA is hybridized to an oligonucleotide array consisting of universal and species-specific 16S rRNA probes.

This method can quickly analyze up to ten thousand samples simultaneously (Kirk and others 2004; Medlin, Groben, and Valentin 2002). Limitations of this technique are that it requires expensive devices and time consuming purification of nucleic acid. A microarray can only be used to map microbial diversity of known species, and it is not possible to discover a new species with this method (Kirk and others 2004).

4.2.2. Polymerase Chain Reaction

As previously described, 5S, 16S, or 23S rRNA gene sequences play a significant role in the study of microbial diversity. The PCR amplified rRNA gene sequences can be used to study microbial diversity using different techniques. There are, however, procedural biases attached to PCR-based molecular techniques. Sample collection, cell lysis, DNA extraction, PCR amplification, and cloning can generate biases. Therefore, it is important to try and minimize these biases while performing each step of the experiment. To eliminate sample collection biases, the samples should be collected from multiple locations on the site, because there is a formation of different microhabitats in

the soil. For example, this occurs in zones where organic matter or animal waste accumulate or in the rhizosphere (Nannipieri 2003). Microbial communities of rhizosphere zones are a result of mutual existence of microbes with plants. Therefore, microbial diversity in rhizosphere will be different from that of bulk soil (Grundmann and Gourbiere 1999; Nannipieri 2003). To eliminate cell lysis biases, it is important to use a suitable method to extract nucleic acid from bacterial cells. An enzyme lysis may not free nucleic acid from all Gram-positive bacteria, and strong sonication might shear the nucleic acid facilitating chimera formation.

Techniques that use combined mechanical and chemical cell lysis can be helpful to reduce biases. For example, in the bead milling technique, glass beads combined with lysis solution, are used to disrupt the cell walls of most gram positive and gram negative bacteria. PCR biases can be introduced, due to presence of DNA templates from the abundant organisms, which will be more frequently available to primers for amplification. To eliminate these biases, PCR products of 3-4 PCRs should be pooled together (Weidner, Arnold, and Puhler 1996). Additionally, chimeric sequences are formed during PCR as a result of contiguous gene amplification from two or more different taxons. Incompletely extended primers switch the template and start extending, thus producing sequences that cannot be placed in a proper phylogenetic group (Cole and others 2005). To eliminate this, a chimera test should be done by sequencing the fragment of interest and comparing the sequences with the sequences in RDP database (Kassen and Rainey 2004). Some of the major PCR based techniques are described below.

4.2.2.1. Denaturing Gradient Gel Electrophoresis (DGGE)

With this method, DNA fragments of the same size but varying sequences can be separated. Genomic DNA is extracted from the environmental samples, and the 16S rRNA genes are PCR amplified. The PCR amplified 16S rRNA genes are run on a polyacrylamide gel, which has an increasing gradient of denaturant or temperature. The denaturants can be formamide or urea. The DNA strand starts to melt as it passes through the denaturant or the temperature gradient (Muyzer and others 1993; Nubel and others 1999). The melting of DNA decreases its mobility. The mobility of fully helical DNA is greater than that of partly melted DNA fragments. Eventually, the melted DNA fragments will stop moving through the gel. The melting point of different base pair sequences will vary, thereby forming separate bands for each species. A 35 to 40 bp GC clamp is placed at the 5′ end of the forward primer in order to keep some part of DNA double stranded during the denaturing process. This will lead to the formation of stable, partially melted DNA molecules. To identify the species, bands are transferred to a nylon membrane and probed with group-specific or species-specific probes (Muyzer and others 1993; Nubel and others 1999). The banding patterns obtained are not affected by the method of DNA extraction, and lesser abundant species are also detected (Maarit-Niemi and others 2001).

DGGE is relatively inexpensive and has high resolution. It can separate sequences differing by even a single base pair (Miller and others 1999). However, the

results obtained will have some biases of the PCR technique, and this method requires special equipment (Kassen and Rainey 2004).

4.2.2.2. Restriction Fragment Length Polymorphism (RFLP)/Amplified Ribosomal DNA Restriction Analysis (ARDRA)

RFLP, also called ARDRA, is another PCR-based technique for diversity studies (Weidner, Arnold, and Puhler 1996). This technique exploits variations in rDNA sequences by digesting 23S, 16S, or 5S rDNA sequences with restriction enzymes to obtain unique patterns for each species.

To study community differences using the RFLP method, total genomic DNA is extracted from the environmental samples (Martin-Laurent and others 2001). Bacterial rDNA sequences (mostly 16S rDNA) are PCR amplified from the genomic DNA using universal primers. The PCR products (e.g., 1.5 kb fragment for bacterial 16S rDNA) are analyzed on 1-1.2% agarose gel. Subsequently, bacterial rDNA clone libraries are constructed by ligating the PCR products into a vector (e.g. pGEM-T) and transforming the recombinant plasmid into competent cells (Weidner, Arnold, and Puhler 1996).

Transformants carrying recombinant plasmids are used to establish clone libraries. Surveys of literature show that the number of clones analyzed for soil microbial community analysis, using the RFLP method, range from as low as 37 to as high as 850 (Dunbar and other 1999; Moyer, Dobbs, and Karl 1994; Weidner, Arnold, and Puhler 1996). The inserts from the recombinant plasmid can be isolated either by colony PCR or by using suitable restriction enzymes.

The isolated inserts are then digested with restriction enzymes. Use of tetrameric restriction enzymes (recognizing four base pairs) is preferred over hexameric in order to increase the chances of obtaining unique RFLP patterns (Moyer, Dobbs, and Karl 1994; Weidner, Arnold, and Puhler 1996). This is because enzymes recognizing four base pairs will cut more frequently than enzymes recognizing six base pair (Moyer, Dobbs, and Karl 1994; Weidner, Arnold, and Puhler 1996). The digestion can be a double digest (e.g. *Hae*III+*Msp*I or *Alu*I+*Pst*I). Otherwise, the digestion can be two separate digests (e.g. any two from *Rsa*I, *Msp*I, *Hin*f, and *Hpa*II).

The restriction digested fragments give a pattern of bands when gel electrophoresis is performed and are detected by ethidium bromide or SYBR green stain. The gels used in the electrophoresis can be either agarose gel or polyacrylamide gel (Chandler, Fredrickson, and Brockman 1997; Hodson and other 1995). The large fragments (500 bp – 40 kb) are resolved by TAE agarose gel. Smaller fragments (50 bp – 1000 bp) can me resolved on Nusieve or MetaPhor agarose gel. Polyacrylamide gel gives higher resolution than any of the agarose gels, however, it is significantly less convenient to prepare than agarose gels (Woodford and Johnson 1998).

The number of different patterns obtained is a function of the diversity of that community. Each pattern can be classified as a single Operational Taxonomic Unit (OTU) (Moyer, Dobbs, and Karl 1994); where a single OTU represents a single bacterial species. The number of OTUs obtained estimates the richness of the community. Numbers of rDNA clones present in a single OTU estimates the evenness of each species.

Structural changes of a community can be detected by this method, but specific phylogenetic groups cannot be identified (Moyer, Dobbs, and Karl 1994). To determine specific phylogenetic groups, one clone from each different OTU needs to be sequenced. The RFLP method is affected by PCR biases and the choice of the enzyme. The choice of enzyme affects the resolution of the method. It is difficult to choose the right enzyme if rDNA sequences are not known (Cole and others 2005). However, this is the least expensive method for studying differences in microbial diversity (Kirk and others 2004).

4.2.2.3. Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is based on the same concept as RFLP, except primers used to amplify the gene are fluorescently labeled at 5′ends to obtain labeled PCR products. The PCR products are cut with restriction endonucleases, mostly by four base pair recognizing enzymes. Distance to the first restriction site from the labeled primer is unique for each species (Kent and others 2003). The restriction digested fragments are run on a sequencing gel or an automated DNA sequence analyzer (e.g. ABI 377 from Applied Biosystems) which separates the terminal restriction fragment sizes. Predicted terminal fragments are compared against a 16S rRNA sequence database known as the RDP database, using the T-RFLP matching software (Kent and others 2003).

This technique overcomes the problem of multiple restriction sites for the same enzyme (Ovreas and others 2003). PCR biases affect this method, however. Also, if the sequences are not known, it becomes difficult to choose which restriction enzyme to use.

In addition, some species have equal distances to the first restriction site (Kassen and Rainey 2004).

4.3. Quantifying Microbial Diversity

Microbial diversity is a measure of species richness and evenness. Many different diversity indexes that incorporate richness and evenness are used to estimate the degree of certainty or uncertainty of the presence of a particular organism in a community. The diversity index most commonly used by biologists is the Shannon Weaver diversity index, which estimates the degree of uncertainty attached to a single species in a community. It is represented as:

$$H = -\Sigma p_i \ln p_i$$

H is the diversity index, and i represents an index number for each species present in a sample; p_i is the number of individuals within a species (n_i) divided by the total number of individuals (N) present in the entire sample (Nubel and others 1999). If there is only a single species present in a community, then the Shannon Weaver diversity index will be zero. This means that there is no uncertainty. Therefore, the diversity index increases with the increase of species richness and species evenness (Nubel and others 1999).

5. Conclusion

Molecular techniques have undoubtedly given new vistas to microbial diversity studies. Culture techniques, however, still have their own importance. These techniques

allow researchers to understand the structure and function of microorganisms and to develop novel molecular techniques (Dahllof 2000).

Our goal is to perform a proof-of-concept study of the differences between the microbial communities of a less restored marsh (<8 y) to those of a more restored marsh (>20 y), using the RFLP method. We hypothesize that by comparing the bacterial diversity of a more disturbed marsh to a less disturbed marsh, one will better be able to characterize the less restored marsh as well as the process of restoration.

Microorganisms play an important role in the ecosystem processes, like decomposition, energy flow, and disease. Quantifying the microbial diversity of salt marshes will provide detailed information regarding their microbial community and community dynamics. By comparing microbial diversity of a less restored with a restored marsh one will gain substantial insight into variation in microbial community structures, the complexity of interactions, and the processes affecting diversity of microbial species in the two marshes (Kassen and Rainey 2004).

Restoring salt marshes will create a critical habitat for many plants and animals. Knowledge of the microbial community structures of these two marshes is one of the many steps to alter the working of the marsh towards creating a fully-restored marsh. To our knowledge, this is the first molecular study of soil bacterial diversity of salt marshes. Here we report preliminary results for soil bacterial diversity of Charleston Slough and Mountain View salt marshes, located in Mountain View, California.

MATERIALS AND METHODS

1. Sample collection

Soil samples were collected in June 2002 from Charleston Slough and Mountain View salt marshes, located in Mountain View, California. Samples were collected from the middle of the marsh. Five individual samples from each marsh were randomly obtained at one location from depths of 5 cm below pickle weed grass, using soil cores of 5 cm diameter. Samples were transferred to sterile sample bottles and transported to the lab on ice. The bottles containing the samples were stored at 4 °C until they were analyzed.

2. Genomic DNA extraction

Microbial DNA was extracted from soil samples using UltraClean Soil DNA Isolation kit (Mo Bio Labs, Carlsbad, California) within 24 h of soil sample collection using the alternative protocol for maximum yield. First, one composite samples for each marsh was established by weighing out 1 g of soil from each of the five Charleston Slough and Mountain View salt marshes' soil samples and mixing them together. From the composite soil samples 1 g of soil was weighed out and transferred into tubes containing 2 ml Mo Bio bead lysis solution, and the tubes were gently vortexed. Solution S1, a cell lysis solution (60 µl), was added. The S1 solution was mixed by inverting the tubes approximately 25 times and then vortexing them for 15 s. Subsequently, 200 µl of Inhibitor Removal Solution (IRS) was added. IRS is a component of the UltraClean Soil DNA Isolation Kit that inactivates phenolic compounds present in soil organic matter and

plant tissues, which inhibit PCR reactions. Bead tubes were secured horizontally on a VSM3 mixer vortex (Shelton Scientific, Peosta, Iowa) and vortexed at maximum speed for 10 min. The tubes were then centrifuged at 10,000 x g for 30 s. The supernatants obtained for both the marshes (545 µl from Charleston Slough and 560 µl from Mountain View) were transferred into new 2 ml microfuge tubes each and 1.3 ml of S3 solution was added into each tube. S3 is a DNA binding salt solution provided by the kit. Both tubes were vortexed for 5 s. Subsequently, a mixture of DNA binding solution and the supernatant were loaded on a spin filter column to allow the DNA to bind to the column and allow all of the impurities to pass through. A washing step with an ethanol-based solution S4 was performed by adding 300 µl of solution S4 and centrifuging for 30 s at 10,000 x g. The flow-through was discarded, and the tubes were centrifuged again for 60 s. The spin filters were placed in new, clean tubes, and 50 µl of S5 solution was used to release the DNA by centrifuging the filters for 30 s at 10,000 x g. The spin filters were then discarded. The isolated genomic DNA from both the marshes were visualized by electrophoresis using a 0.8% agarose gel (Fisher Scientific, Rochester, New York). λ *Hin*dIII was used as molecular weight marker. The gel ran at 70 V for 90 min and was then stained with ethidium bromide (1 µg/ml) for 15 min. The DNA was quantified by Jenway UV spectrophotometer. Absorbencies were obtained at O.D. 260. The genomic DNA obtained was stored at -20 °C. The DNA solution was used directly for PCR without any dilution.

3. Polymerase chain reaction

PCR amplification of the 16S rRNA gene from bacterial genomic DNA samples of Charleston Slough and Mountain View salt marshes were carried out using universal primers. Universal primers fD1 and rD1 published by Weisburg and others (1991) were used to amplify bacterial 16S rRNA genes. The primers were synthesized by Qiagen (Qiagen, Valencia, California). Identical PCR conditions were applied to amplify the 16S rRNA gene from both the marsh samples, with few exceptions. The PCR reaction mixture included 2 µl of 10x PCR buffer, 2 mM of $MgCl_2$, 0.5 mM dNTP, 0.5 µl (10 u/µl) of Taq polymerase (all components from Promega, San Luis Obispo, California), 0.5 µM of fd1, 0.5 µM of rd1, 74 ng of DNA template in a total volume of 20 µl. The reaction was incubated in a PT-thermocycler (MJ Research, Inc., Watertown, Massachusetts). The cycling program used was 94 °C for 9 min (initial denaturing), 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 3 min (extension), 72 °C for 10 min (final extension), 4 °C forever (hold). To optimize the Charleston Slough PCR reaction, the final $MgCl_2$ concentration was 3 mM, instead of 2 mM; and the annealing temperature was increased to 60 °C.

Two aliquots each of 10 µl of Mountain View and 10 µl of Charleston Slough PCR amplified products were run on 1% Tris-acetate-EDTA (TAE)-agarose gel (Fisher Scientific, Rochester, New York) at 70 V for 90 min. The gel was stained with ethidium bromide 1µg /ml for 15 min. Single bands of 1.5 kb PCR products obtained for both the marshes were cut out from the agarose gel and purified using a Qiagen Mini Elute kit (Qiagen, Valencia, California). The DNA was eluted with 30 µl of elution buffer

provided with the kit (Qiagen, Valencia, California). The concentration of PCR products were estimated using DNA mass standards run on 1% agarose gel (Fisher Scientific, Rochester, New York) with 10 µl of each gel purified PCR products.

4. Construction of Bacterial 16S rDNA Clone Library

The gel purified 16S rDNA PCR products were ligated into pGEM-T vector using pGEM-T vector system I (Promega, San Luis Obispo, California). Figure 2 shows a map of the pGEM-T vector. The ligation reactions were carried out in 0.5 ml tubes that contained 50 ng of insert DNA, 1 µl of pGEM-T vector (50 ng/µl), 5 µl of ligase buffer, and 1µl of T4 DNA Ligase (3 Weiss units/µl, Promega, San Luis Obispo, California), in a final volume of 10 µl. The reactions were incubated at 4 °C for 20 h. Two independent ligation reactions for each marsh were pooled together and transformed into commercially-prepared JM109 competent cells (Promega, San Luis Obispo, California), according to the manufacturer protocol.

The transformed cells were grown on Luria Broth (LB) plates containing X-gal (80 µg/ml), IPTG (0.5 mM) and ampicillin (100 µg/ml). 100 µl of 1:1 and 1:100 dilutions were plated. The plates were incubated overnight at 37 °C. Control reactions were performed to ensure that the ligase buffer and ligase enzyme used in these experiments were active. A positive control ligation reaction was processed using a control DNA provided with the pGEM-T kit. This control ligation reaction was processed in parallel with the experimental samples and transformed into competent cells, then plated on ampicillin, IPTG/X-GAL plates.

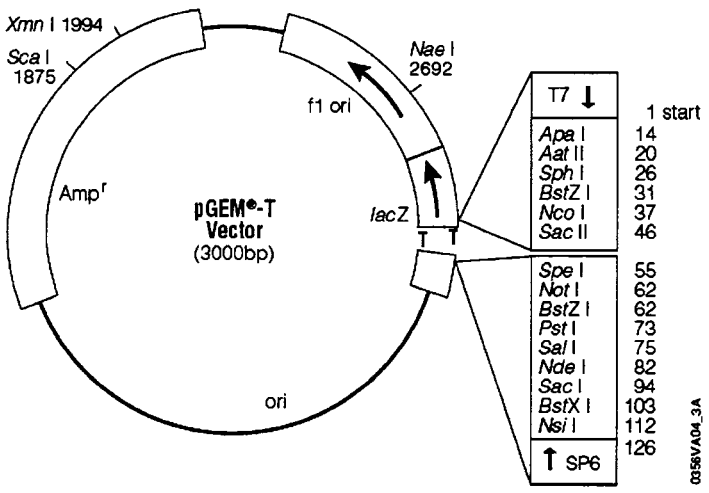

Figure 2. Map of pGEM-T vector (Promega, San Luis Obispo, California).

plated on ampicillin IPTG/X-GAL plates. Super-coiled plasmid control DNA was provided with the JM109 cells. This super-coiled plasmid DNA was used (as per the manufacturer instructions) as a control to estimate the transformation efficiency of the competent cells.

White colonies, 100 from each transformation, were picked and grown overnight at 37 °C in 6 ml LB medium, containing ampicillin (100 µg/ml). Broth (1 ml) from each culture tube was put into cryovials, and 70 µl of Dimethyl Sulphoxide (DMSO) was added. The broth and DMSO suspension was kept at -70 °C after 10 min incubation at room temperature.

Plasmid preps for the 100 Charleston Slough and 100 Mountain View salt marshes' clones were performed on the remaining 5 ml broth using the Wizard Miniprep DNA Purification System (Qiagen, Valencia, California), as per the instruction manual using the protocol, which uses a manifold vacuum pump (Promega, San Luis Obispo,

California). Spectrophotometric analysis was done for all the plasmid preps using a Jenway spectrophotometer, to determine the amount of DNA.

A *Bst*ZI restriction enzyme was used to cut out the inserts from the recombinant plasmids. All the reaction mixtures contained 2.5-3 μg of plasmid prep DNA, 1 μl of *Bst*ZI enzyme (10 u/μl), 4 μl of buffer D, 0.4 μl of BSA (Promega, San Luis Obispo, California) to the total of 40 μl reaction volume. The reaction mixture was incubated at 50 °C for 2 h. The whole reaction mixture was run on 1% TAE-Agarose gel at 70 V for 90 min with a 1 kb DNA ladder (Promega, San Luis Obispo, California) as a molecular weight marker. Subsequently, the gels were stained with ethidium bromide (1 μg/10 μl), and a Bio-Rad Geldoc analyzer system was used to screen for the presence of 1.5 kb insert and to capture images. A total of 45 clones exhibited the presence of 1.5 kb 16S rRNA gene fragments. The 1.5 kb fragments were excised from the agarose gel and were purified using a Minielute Gel Purification kit (Qiagen, Valencia, California). The DNA was eluted in 50 μl of nuclease-free water. The amount of 1.5 kb DNA in this 50 μl of nuclease-free water was estimated by the following calculation. The insert (1.5 kb) was one-third and the vector (3 kb) was two-thirds of the total recombinant plasmid. If 3 μg of the plasmid was cut, it contained 1 μg of insert and 2 μg of vector DNA. Assuming 50% recovery after gel purification, the Minielute Gel Purification 50 μl elution contained 500 ng of 1.5 kb insert DNA.

5. 16S rDNA RFLP Analysis

The 1.5 kb gel extracted 16S rRNA gene fragments from each of the clones were restricted using four base pair recognizing restriction enzymes the tetrameric endonucleases *Msp*I and *Hae*III. Table 1 describes the characteristics of the two enzymes. These enzymes have previously been shown to cut 16S rDNA sequences into fragments that allow optimal resolution (Moyer, Dobbs, and Karl 1994; Weidner, Arnold, and Puhler 1996). First, the 1.5 kb fragments obtained from 25 Mountain View and 20 Charleston Slough salt marshes' clones were digested with *Msp*I (10 u/µl, Promega, San Luis Obispo, CA), and then with *Hae*III (10 u/µl, Promega, San Luis Obispo, California). The method for obtaining 1.5 kb 16S rRNA gene fragments from recombinant clones for restriction was exactly the same as described on the previous page for both restriction enzymes; except that, for the *Hae*III enzyme, the cultures for plasmid preps were revived from the cells kept at -70 °C. The digestion reactions contained 17 µl of DNA (7 µg/µl to 10 µg/µl), 0.5 µl of *Hae*III or *Msp*I enzyme, 2 µl of buffer C (Promega, San Luis Obispo, CA) for *Hae*III and buffer B (Promega, San Luis Obispo, California) for *Msp*I, and 0.2 µl of Bovine Serum Albumin (Promega, San Luis Obispo, California).

Table 1. Characteristics of *Msp*I and *Hae*III enzymes.

Name	Sequence	Site Length	Overhang
*Msp*I	CCGG	4	five prime
*Hae*III	GGCC	4	blunt

The total volume of all the reactions was 20 µl. The restriction digest was performed at 37 °C for both the enzymes for a minimum of 1 h incubation. The majority of the inserts were fully digested within 1 h of incubation. For the other fragments, the incubation period was increased until the fragments were fully digested or a maximum incubation period of 24 h. The resulting RFLP products were separated using a 2% MetaPhor gel, stained with 0.5 µg/µl of ethidium bromide run within the gel, and visualized under the UV light using the Bio-Rad Geldoc analyzer system.

6. 2% MetaPhor Agarose Gel Preparation

A TAE buffer (100 ml) was poured into a flask and incubated at 4 °C for 10-15 min. MetaPhor agarose (2 gm) was then added to the cold buffer. A clean stir bar was put in the cold TAE buffer and stirred. Slowly, the agarose was added to the stirring TAE. The slow addition of MetaPhor agarose prevented frothing. The MetaPhor agarose TAE mixture was soaked for another 10-15 min before heating it in the microwave, in order to completely mix the buffer and agarose. This also helps to prevent foaming. The melted agarose was then allowed to cool until it reached 65 °C. Ethidium bromide was then added into the mixture (0.5 µg/ml). All gels were run at 65 V for approximately 2 h and 15 min. After the run, the gel was cooled (4 °C for 10-15 min) to decrease fragility.

RESULTS

1. Soil Genomic DNA and 16S rDNA Amplification

Mountain View and Charleston Slough salt marsh soils yielded 4 μg and 1.625 μg of genomic DNA per gram of soil sample respectively. The genomic DNA extraction was carried out once for each marsh. Single bands of 1.5 kb were obtained from the genomic DNA for both the marshes (Figure 3). The negative control showed no amplification.

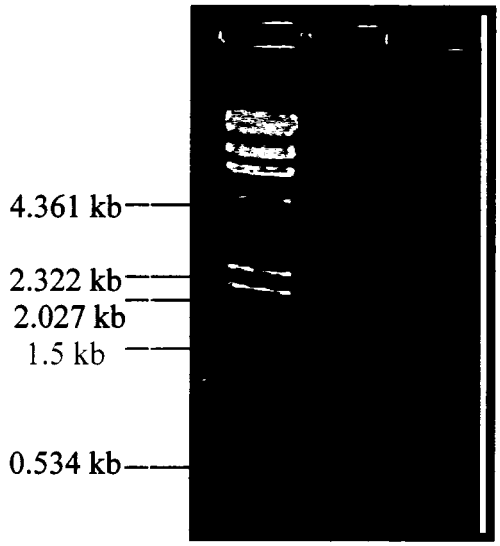

Figure 3. Gel picture of PCR amplified 1.5 kb 16S rRNA gene from soil genomic DNA of Mountain View and Charleston Slough salt marshes. TAE Agarose gel electrophoresis (1%). Lane 1, λ *Hin*dIII molecular weight marker from Promega; Lane 2, Mountain View PCR fragments; Lane 3, Charleston Slough PCR fragments.

2. Mountain View Clone Library

From the isolated microbial genomic DNA, 1.5 kb rDNA genes were amplified by PCR, band purified, cloned into pGEM-T vector as described in materials and methods. A total of 100 white clones were selected by blue/white screening, and 25 exhibited the presence of 1.5 kb insert after cutting the recombinant plasmid with a *Bst*ZI enzyme and analyzing on 1% agarose gel. Each clone was numbered sequentially as it was analyzed for the presence of insert. Figure 4 shows one of the gel pictures of recombinant plasmids (4.5 kb) when cut with *Bst*ZI enzyme produce 1.5 kb insert and 3 kb plasmid DNA.

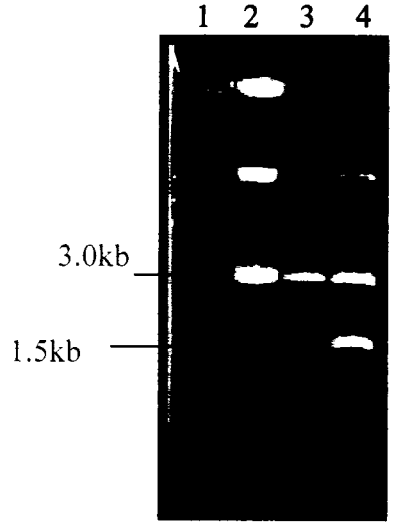

Figure 4. Gel picture showing 1.5 kb insert cut from 3 kb plasmid of Mountain View salt marsh clones using *Bst*ZI enzyme. Lane1, 1 kb DNA ladder. Lane 2, 3 and 4 contain plasmid (extracted by plasmid preps) cut with *Bst*ZI. Lane 4 shows both 1.5 kb fragment and 3 kb plasmid. Lane 2 and 3 shows only 3 kb plasmid.

Restriction of complete 16S rDNA inserts was performed with *Msp*I and *Hae*III enzymes to identify discrete OTUs. Each discrete RFLP pattern, which was either unique for a single clone or similar for two or more clones, represented a single OTU. For Mountain View salt marsh 11 OTUs were detected after restriction of the entire 16S rDNA clone library with *Msp*I. Restriction of all 16S rDNA clones with *Hae*III was slightly more discriminating as 2 additional OTUs were detected; OTU MV4 was separated from MV5, and MV11 was separated from MV12 in this manner. Overall, a total of 13 OTUs were detected (Table 2; Figure 5 and Figure 6).

Table 2. Number of clones in each unique Mountain View salt marsh OTU.

MspI digest (Figure 5)	Number of clones for each unique pattern obtained by *MspI*	*Hae*III digest (Figure 6)	Number of clones for each unique pattern obtained by *Hae*III	OTU (patterns)	Number of clones for each OTU
Lane #1	2	no extra patterns	2	MV 1	2
Lane # 2	1			MV 2	1
Lane # 3	1			MV 3	1
Lane # 4	5	2 different patterns (i)	2	MV 4	2
		(ii)	3	MV 5	3
Lane # 6	2	no extra patterns	2	MV 6	2
Lane # 7	1			MV 7	1
Lane # 8	2	no extra patterns	2	MV 8	2
Lane # 9	2	no extra patterns	2	MV 9	2
Lane # 11	3	no extra patterns	3	MV 10	3
Lane #13	2	2 different patterns (i)	1	MV 11	1
		(ii)	1	MV 12	1
Lane # 14	4	no extra patterns	4	MV 13	4

Figure 5. Composite gel picture of Mountain View *Msp*I digests. RFLP patterns obtained on 2% MetaPhor agarose gel. 1.5 kb 16S rDNA of Mountain View clone library were cut with *Msp*I enzyme. Lane 5, 10, 12 and 15 are size marker 1 kb ladder lanes. The red arrows point to the 1.5 kb band of each size marker. Eleven unique patterns are obtained from 25 different clones. Lane1:MV64; Lane 2: MV11; Lane 3: MV29; Lane 4: MV33; Lane 6: MV 76; Lane 7: MV97; Lane 8: MV2; Lane 9: MV56; Lane 11: MV62; Lane 13: MV67; Lane 14: MV17.

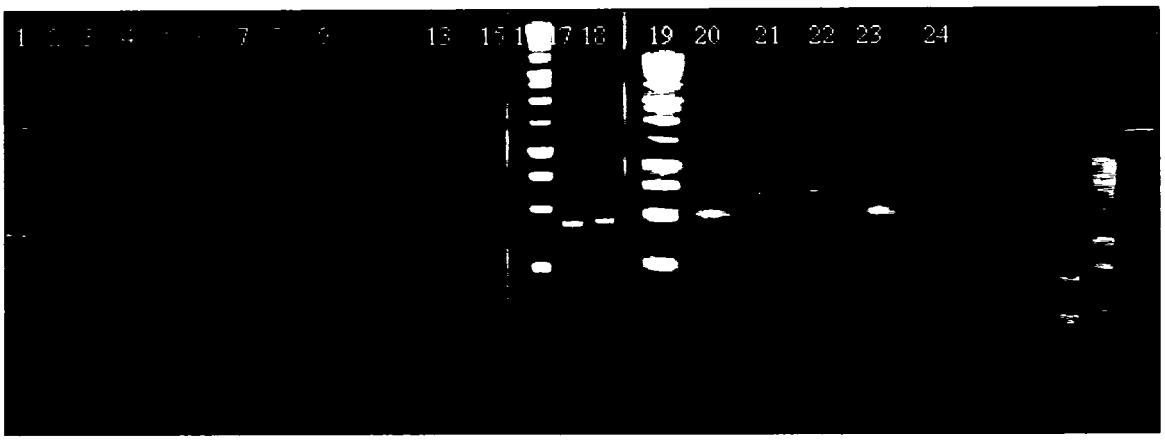

Figure 6. Composite gel Picture of Mountain View *Hae*III digests. RFLP patterns are obtained on 2% MetaPhor agarose gel. 16S rDNA of Mountain View salt marsh clone library were cut with *Hae*III enzyme. Lane 1, 7, 13, 16, 19 and 28 are size marker 1 kb ladder lanes. The red arrows point to the 1.5 kb band of each size marker. Below is each lane with the Mountain View salt marsh clone number.
Same patterns are obtained for the clones put inside parenthesis, when 1.5 kb fragments are digested with *Msp*I enzyme:-
(Lane 2: MV39; Lane 4: MV 76)
(Lane 3:MV97; Lane 14: MV49);
(Lane 5: MV56; Lane 6: MV86);
(Lane 8: MV51; Lane 9: MV87; Lane 10: MV23; Lane 11: MV42; Lane 12: MV2);
(Lane 15: MV29; Lane 21: MV7; Lane 24: MV53)
(Lane 17: MV2; Lane 18: MV32);
(Lane 20: MV67; Lane 23: MV22);
(Lane 22: MV33; Lane 25: MV 78; Lane 26: MV80; Lane 27: MV 75; Lane 29: MV48)

3. Charleston Slough Clone Library

A total of 100 white clones were picked using blue/white screening, and 20 exhibited the presence of 1.5 kb inserts after the recombinant plasmid was cut with *Bst*ZI. Restriction of complete 16S rDNA inserts was performed with *Msp*I and *Hae*III enzymes to identify discrete OTUs. For Charleston Slough salt marsh 7 OTUs were detected after restriction of the entire 16S rDNA clone library with *Msp*I. Restriction of 16S rDNA clones with *Hae*III generated 3 additional OTUs; OTU CS1, CS2, CS3, and CS4 were

differentiated in this manner. Overall 11 OTUs were detected (Table 3; Figure 7 and Figure 8).

Table 3. Number of clones in each unique Charleston Slough OTU.

MspI digest (Figure 7)	Number of clones for each unique pattern obtained by *Msp*I digestion	*Hae*III digest (Figure 8)	Number of clones for each unique pattern obtained by *Hae*III digestion	OTU (patterns)	Number of clones for each OTU
Lane #1	14	4 different patterns (i)	5	CS 1	7
		(ii)	6	CS 2	3
		(iii)	2	CS 3	2
		(iv)	1	CS4	1
		(v)	1	CS5	1
Lane # 3	1			CS6	1
Lane #4	1			CS 7	1
Lane #5	1			CS 8	1
Lane #6	1			CS 9	1
Lane #7	1			CS 10	1
Lane #8	1			CS 11	1

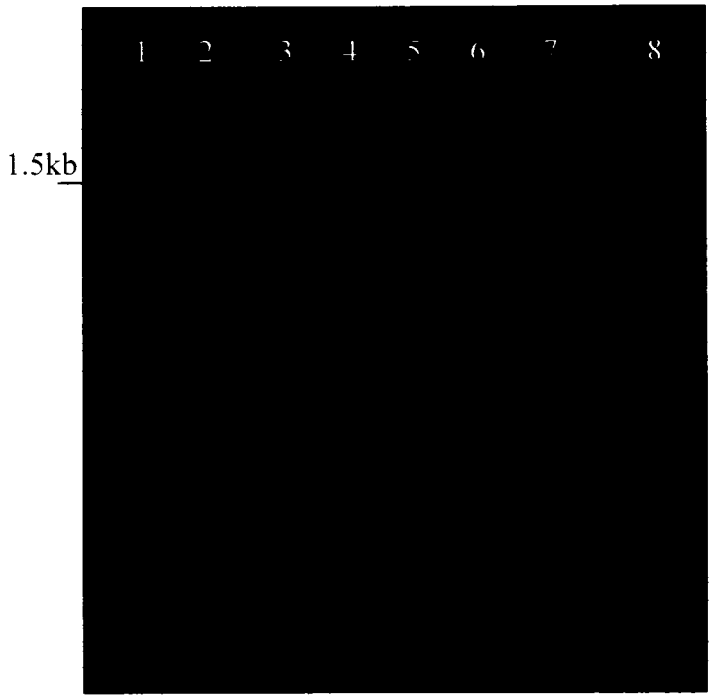

Figure 7. Composite gel Picture of Charleston Slough *Msp*I digests. RFLP patterns are obtained on 2% MetaPhor agarose gel. 16S rDNA of Charleston Slough clone library were cut with an *Msp*I enzyme. Lane 2 is molecular weight marker (1 kb ladder). Seven unique patterns are obtained. The red arrows point to 1.5 kb band of each size marker. Lane 1: CS6; Lane 3: CS13; Lane 4: CS86; Lane 5: CS36; Lane 6: CS51; Lane 7: CS72.

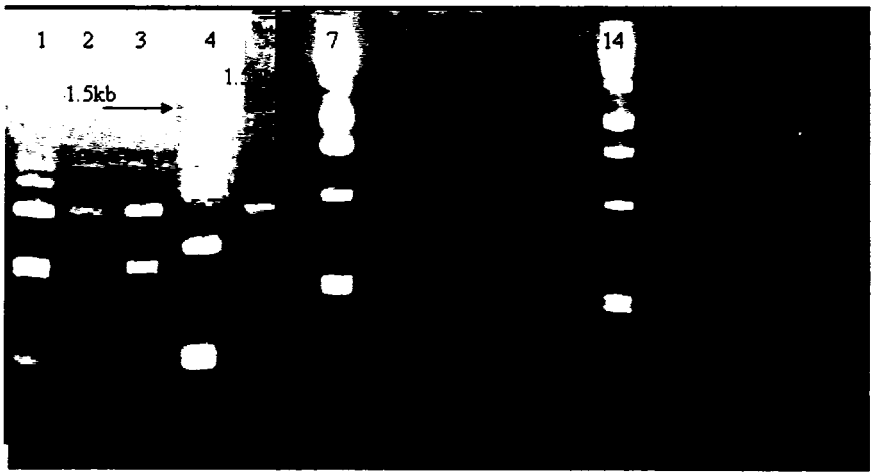

Figure 8. Composite gel Picture of Charleston Slough *Hae*III digests. RFLP patterns obtained on 2% MetaPhor agarose gel. 16S rDNA of Charleston Slough clone library were cut with *Hae*III enzyme. Lane 4, 7, and 14 are molecular weight marker (1 kb ladder). Lane 16, 17, and 19 are empty.
Same patterns are obtained for the clones put inside parenthesis when 1.5 kb fragments are digested with *Msp*I enzyme:-
(Lane 1: CS6; Lane 2: CS18; Lane 3: CS42; Lane 5: CS98; Lane 6: CS 90; Lane 13: CS59; Lane 15: CS24)
(Lane 8: CS43; Lane 10:CS66; Lane 11:CS74)
(Lane 9: CS78; Lane 12: CS80)
(Lane18: CS92)
(Lane 20: CS28)

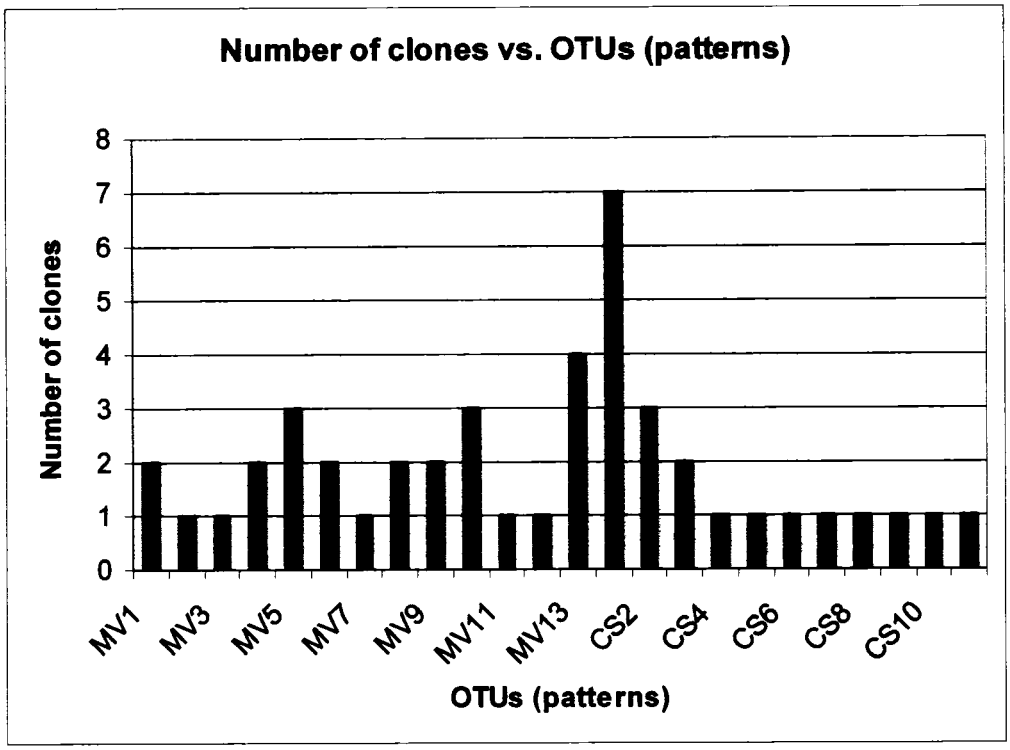

Figure 9. Plot showing Number of Clones vs. OTUs. Distribution of OTUs of 45 bacterial 16S rDNA clones from Mountain View and Charleston Slough salt marshes in Mountain View, California. Abundance, as determined by the number of 16S rDNA clones found in each OTU, was used to define community structure.

The Shannon-Weaver diversity indexes of the Mountain View and Charleston Slough salt marshes were 2.4 and 1.9 respectively (Table 4). The dominant OTU comprised of 65% (20 clones) for Charleston Slough and 42% (25 clones) for Mountain View clone libraries (Figure 9). Dominant OTUs are defined as the species with a higher number of clones than the median for the sample. Two common OTUs were found among the two marshes. Mountain View clones, MV33, MV48, MV78, MV80, and MV75 had same patterns when digested with *Msp*I and *Hae*III as Charleston Slough clones CS6, CS18, CS42, CS98, CS90, CS59, and CS29.

Table 4. Shanon Weaver diversity index calculation sheet.

Diversity index $H = -\Sigma pi \ln pi$

i is an index number for each species present in a sample

pi is the number of individuals within a species (ni) divided by the total number of individuals (N) present in the entire sample

Mountain View salt marsh				Charleston Slough salt marsh			
i	Number of Individuals within a species (ni)	Pi	PilnPi	i	Number of Individuals within a species (ni)	Pi	PilnPi
1	2	0.0800	-0.2021	1	7	0.3500	-0.3674
2	1	0.0400	-0.1288	2	3	0.1500	-0.2846
3	1	0.0400	-0.1288	3	2	0.1000	-0.2303
4	2	0.0800	-0.2021	4	1	0.0500	-0.1498
5	3	0.1200	-0.2544	5	1	0.0500	-0.1498
6	2	0.0800	-0.2021	6	1	0.0500	-0.1498
7	1	0.0400	-0.1288	7	1	0.0500	-0.1498
8	2	0.0800	-0.2021	8	1	0.0500	-0.1498
9	2	0.0800	-0.2021	9	1	0.0500	-0.1498
10	3	0.1200	-0.2544	10	1	0.0500	-0.1498
11	1	0.0400	-0.1288	11	1	0.0500	-0.1498
12	1	0.0400	-0.1288	N	20		-1.9308
13	4	0.1600	-0.2932				
N	25		-2.4561				
$H = -\Sigma pi \ln pi$			2.4561				1.9308

4. Controls

4.1. Ligation Reaction Controls

No colonies were obtained when the negative control ligation reaction was transformed into JM109 cells and plated on X-gal, IPTG, and ampicillin plates. The positive control (reaction with control insert DNA) ligation reaction mixture when transformed into JM109 cells produced 43 blue colonies and 59 white colonies.

4.2. Transformation controls

The first transformation control was to check the efficacy of ampicillin. The JM109 cells were plated with and without selective medium and produced a lawn of colonies and no colonies respectively.

The second transformation control was to check the competent cells' efficiency. The control DNA (0.1 ng) provided by the manufacturer was transformed into the 100 µl of JM109 cells. The transformation reaction was added to 900µl of medium (0.1 ng DNA per ml and the bacteria). From that volume, a 1/10 dilution with medium (0.01 ng DNA/ml) was made and 100 µl was plated (0.001 ng DNA/100 µl). In total, 185 white colonies were obtained. Therefore, the transformation efficiency was 1.85×10^8 cfu/µg: 185 cfu/0.001 ng = 1.85×10^5 cfu/ng = 1.85×10^8 cfu/µg DNA.

DISCUSSION

Estimating microbial diversity using the PCR-RFLP method is a widely-used technology for studying differences in microbial communities. In this proof-of-concept study, we confirm that by molecular methods using bacterial universal primers (Weisburg and others 1991), we can study the soil bacterial populations of salt marsh ecosystems. To our knowledge, this is the first molecular survey for the estimation of soil microbial diversity of salt marshes. We estimated soil bacterial diversity of Charleston Slough and Mountain View salt marshes by determining the bacterial species richness and evenness with 45 clones.

1. Method Optimizations and Analysis

Salt marshes serve as the depositories for large amounts of organic matter. Humic acid pigments are often extracted with genomic DNA and can inhibit subsequent PCR amplification (Kent and others 2003). Analysis of the microbial community structure using the RFLP method requires that the extracted DNA be of a sufficient quality for subsequent PCR. We were successful in obtaining single bands of 1.5 kb PCR amplified DNA because the Mo Bio kit selected to extract genomic DNA uses an IRS solution; which prevents the co-extraction of PCR inhibiting substances. Considerable care was taken to reduce biases during the DNA extraction, amplification, and cloning steps. The Mo Bio kit was selected for its bead beating technology, which ensures that gram positive, spore forming, or otherwise recalcitrant microbes are lysed in the genomic DNA extraction. Four replicate PCR reactions were performed for each marsh to avoid

biases attached to any single PCR reaction. Hot PCR start and high denaturing (94 °C) temperatures were applied to increase the specificity of the priming reactions. Highly competent cells (> 10^8 cfu/μg) were used to maximize the number of 16S rDNA clones recovered from PCR amplification. Using highly competent cells, however, did not lead to recovery of more clones with 1.5 kb fragments. None of the white colonies isolated as a result of transformation showed the presence of 1.5 kb insert on the first ligation reaction attempt. Therefore, the ligation reaction was repeated keeping all the conditions the same, but the incubation time and temperature were changed from 1 h at room temperature to 20 h at 4° C. This change resulted in obtaining some white colonies with inserts, compared to none in the previous reaction; but still the white colonies with inserts were few.

All our controls worked well, which determined that the ligase enzyme and the buffers used in the ligation mixture were active. One explanation for obtaining fewer recombinant clones is that the PCR amplified 1.5 kb inserts were overexposed to UV light – leading to a formation of pyrimidine dimers, which interfered with the ligation. Therefore, performing PCR amplification of 1.5 kb rRNA gene from the soil genomic DNA, and taking care not to overexpose the fragments to UV light, may help in obtaining more recombinant clones. Another reason for not obtaining enough white clones with inserts can be that an optimal insert:vector ratio was not obtained. Our insert:vector ratio was 2:1. The ligation reaction protocol suggests that the ratio is between 1:8 and 8:1. Using different insert:vector ratios may help in obtaining a higher number of recombinant clones.

RFLP patterns on the 2% MetaPhor agarose gels are difficult to interpret, due to partial digestion of 1.5 kb fragments (Wu and others 2000). Partial digestion is identified by adding the fragment lengths of each pattern. The sum of the fragment lengths of each pattern greater than 1.5 kb represented partial digestion. To overcome this problem in our experiments, the fragments were digested with *Msp*I and *Hae*III for a longer period of time than recommended by Promega. Increasing the incubation period was helpful; the digests displayed no incomplete cuts except the few seen in Figure 5 lane 17 (clone MV2), lane 18 (clone MV32), lane 26 (clone MV80) and Figure 7 lane 1 (clone CS6), which consistently showed partial digestion. Asakiewicz, Schaefer, and Barkay (2003) suggest solving this problem by adding only the bands of highest intensity. The other approach applied to achieve complete digestion of the inserts was to increase the amount of restriction enzymes (*Msp*I and *Hae*III). However, this did not show any difference in the results.

Another problem in interpreting RFLP patterns on gels are missing fragments; this means the sum of the fragment lengths of each pattern is less than 1.5 kb (Wu and others 2000). In Figure 5, lane 5 (clone MV56) and lane 6 (clone MV86) show single fragments that are approximately 350 bp long. Figure 4, lane 1 (clone MV64) and lane 3 (clone MV29) show more than two fragments; but the fragment lengths do not add up to 1.5 kb. There are two possible explanations for these results. One is that the tetrameric endonucleases have cut the 16S rDNA into very small pieces, which migrate off the gel. The other explanation is that there are repeating sequences present at equal intervals, which are recognized by the enzymes. These equal interval repeating sequences produce

all equally-sized fragments, which are seen as a single band on the gel. To test this explanation, a 16S rDNA gene sequence of *E.coli* was obtained from the National Center of Biotechnology Information (NCBI) website and analyzed (NCBI 2006). Table 5 shows all the recognition sites of *Msp*I and *Hae*III in *E.coli* 16S rDNA sequences – and the respective length of each fragment obtained from the RDP database (Rondon and others 2000). In this analysis we observed that the *E.coli* 16S rDNA sequence does not carry repeating sequences recognized by *Msp*I and *Hae*III, which occur at equal intervals. However, the *Hae*III enzyme does cut the DNA into very small fragments. The largest fragment is 317 bp long, and there are smaller fragments that are 34, 46, and 68 bp. Such small fragments can only be seen on the gel when increased DNA concentrations (> 400 ng) are loaded on the gel or with more sensitive stains like Syber-Green (Wu and others 2000).

Syber-Green is 25 times more sensitive than ethidium bromide stain for double stranded DNA. It can detect as low as 60 pg of DNA when illuminated with UV light (Molecular Probe. Inc. 2003). It is difficult to analyze the RFLP patterns visually, and the task becomes more difficult when the number of patterns increases; therefore, analysis using software like BioImage system and GelCompar system would have been more useful than a visual analysis. In GelCompar system, gel images saved as TIFF files are converted and normalized with a molecular-sized marker and analyzed with GelCompar software (Gerner-Smidt and others 1998).

Table 5. *Msp*I and *Hae*III cutting sites of *E.coli* 1.5 kb 16S rDNA sequences and fragment lengths.

	1.5 kb fragments cut at the following sites	Each fragment length (bp)	Total number of fragments generated from respective enzymes
*Msp*I	502	502	
	612	110	
	718	106	
	855	137	8 fragments
	1135	280	
	1139	4	
	1301	162	
	1382	81	
*Hae*III	46	46	
	212	166	
	416	204	
	733	317	
	894	161	9 fragments
	928	34	
	1138	210	
	1206	68	
	1386	180	

2. Community Richness and Evenness

Atlas and others (1991) found that physical characteristics affect the microbial diversity of a community. Lower soil microbial diversities were recorded on disturbed sites (presence of chemical pollutant) compared to undisturbed sites. Atlas and others (1991) also found that few species dominate in the disturbed area because these species have increased their physiological tolerance and can compete successfully within a

disturbed community. The Charleston Slough salt marsh is more disturbed than the Mountain View salt marsh. One example of this disturbance is improper water drainage. Therefore, its microbial diversity is expected to be reduced. However, Shannon-Weaver diversity index (H) values obtained for Mountain View and Charleston Slough salt marshes obtained from our experiments do not demonstrate significant differences between the richness of the two communities. It is difficult to predict whether the diversity value and uneven distribution obtained of the two marshes are a correct representation of the community, or merely a representation of a low number of analyzed clones.

As more clones are analyzed, the number of new OTUs found increases, and eventually the curve reaches an asymptote. Hughes and others (2001) compared data from five communities; soil bacteria, human mouth, tropical birds, tropical moths, and temperate forest to compare the shapes of the accumulation curves. The more concave, downward the curve, the better the community analysis (Hughes and others 2001). To determine how well the total diversity of 16S rDNAs in the libraries of our experiments was captured, accumulation curves (Figure 10) were plotted for both Charleston Slough and Mountain View salt marshes. The shape of the Charleston Slough salt marsh curve was more concave, downward, than the Mountain View salt marsh curve. However, the difference in the shape of the curve was not significant, and none of the curves was near asymptote. Therefore, a higher number of clones need to be analyzed in order to make precise conclusions for the differences in microbial communities between the two marshes.

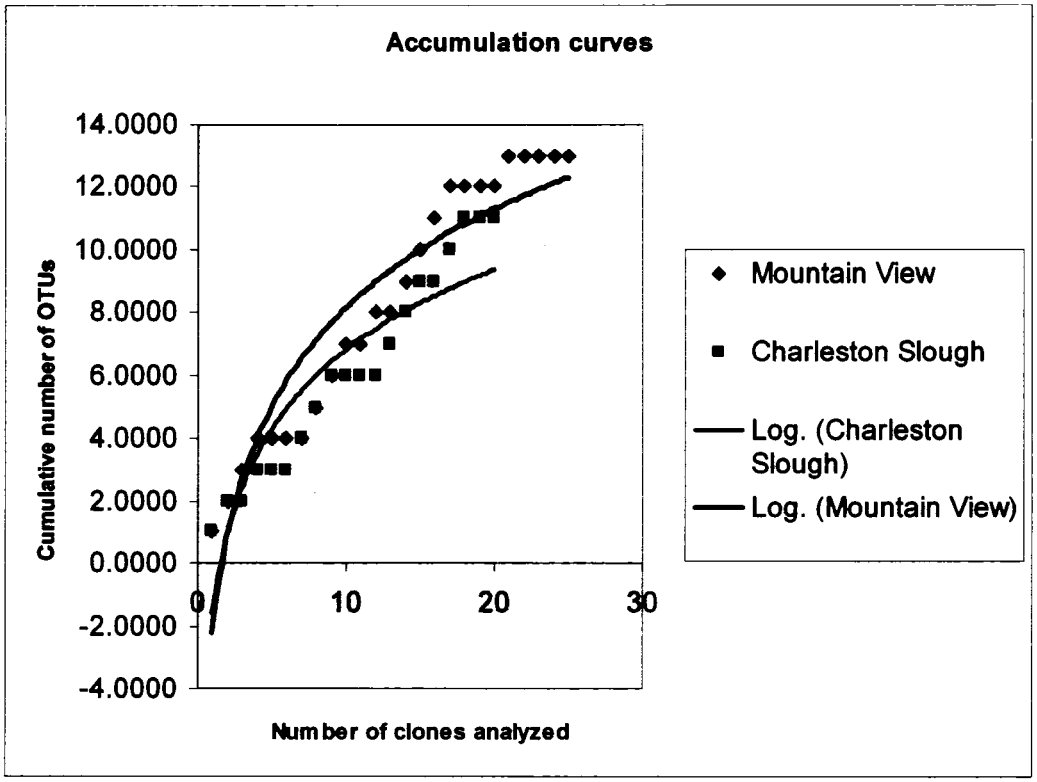

Figure 10. Cumulative number of OTUs plotted as a function of appearance during the sampling of the clones.

3. How Many More Clones Need to be Analyzed ?

Because this is the first molecular survey for estimating the soil bacterial diversity of salt marshes, there are no previous data available to compare our work. To determine the number of different OTUs obtained versus the number of clones analyzed, a literature survey was conducted of other soil microbial diversity studies. Chandler, Fredrickson, and Brockman (1997) compared bacterial communities in two different subsurface soils analyzing 51 and 42 clones and obtained 20 and 25 RFLP patterns. McCaig, Glover, and Prosser (2001) compared bacterial communities in improved and unimproved grassland

samples by sequencing fragments of interest from approximately 135 clones for each grassland and obtained 65 and 77 different species (McCaig, Glover, and Prosser 2001). Palmer and Young (2003) studied rhizobial soil to estimate microbial diversity and obtained 25 different RFLP patterns after analyzing 285 clones. Dunbar and others (1999) compared bacterial community diversity of arid soils by analyzing a total of 801 clones and obtained 498 different RFLP patterns.

In published experiments, none show any fixed number of clones that are sufficient to estimate the microbial diversity of a community. To make precise conclusions regarding the differences among the diversity of soil bacteria in the restored and less restored marshes utilized in our study, it is important to collect more data by analyzing clones, until an asymptote is reached. Many statistical methods (curve extrapolation, parametric, and non parametric approaches) have been developed to estimate community richness using available microbial sample data. Before applying statistical methods in any environmental analysis, anywhere from 200-1000 clone samples are typically required (Hughes and others 2001). A statistical approach should then be used to calculate the number of additional clones required to precisely determine the differences in the community and accurately estimate the total microbial diversity of a community.

For example, McCaig, Glover, and Prosser (2001) compared 16S rRNA clone libraries of fertilized and unfertilized soil by analyzing approximately 135 clones from each soil, and concluded that there is no difference between the microbial communities of the two soils. Curtis, Sloan, and Scannell (2002) used McCaig, Glover, and Prosser

(2001) data to estimate the total microbial diversities using parametric statistical approach and suggested that at least 250 clones from each community need to be analyzed to make precise conclusions regarding the differences among the two communities (Bohannan and Hughes 2003). Curtis, Sloan, and Scannell (2002) also estimated that 6300 different OTUs are present in the soils studied by McCaig, Glover, and Prosser (2001). This means the total OTU obtained by McCaig, Glover, and Prosser (2001) was only 2.25% (142 OTUs) of the actual OTUs present in the soil. Hughes and others (2001) used McCaig, Glover, and Prosser (2001) data to estimate soil bacterial diversity using non-parametric statistical approach and estimated the presence of 597 OTUs in the soil samples. This means the total OTU obtained by McCaig, Glover, and Prosser (2001) was only 20% of the actual OTUs present in the soil (Bohannan and Hughes 2003). Bohannan and Hughes (2003) conclude that regardless of statistical approach used to estimate total microbial diversity of McCaig, Glover, and Prosser (2001) data, it was clear that the sample data was too small to make conclusions regarding the differences in the soil microbial community. This suggests that to make precise conclusions regarding the differences in microbial diversity of the two salt marshes in our study, at least 200 clones from each marsh should be analyzed. Using that data statistically, one will be able to estimate the number of clones needed to be analyzed in order to measure the differences between microbial communities.

4. Further Studies

RFLP is the least expensive method to estimate microbial diversity using molecular methods. Studies show that RFLP can differentiate microorganisms at a species level when using the 16S rRNA gene.

In our work, to prove that all unique patterns represent a single OTU, it is important to sequence one member from each group. Optimization of this RFLP protocol includes increasing the incubation period of tetrameric endonucleases reaction mixture to obtain complete digestion; however, further optimization is required to use this protocol. Once the procedure is fully optimized, the project can be continued with samples collected from different seasons of more restored and less restored marshes.

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